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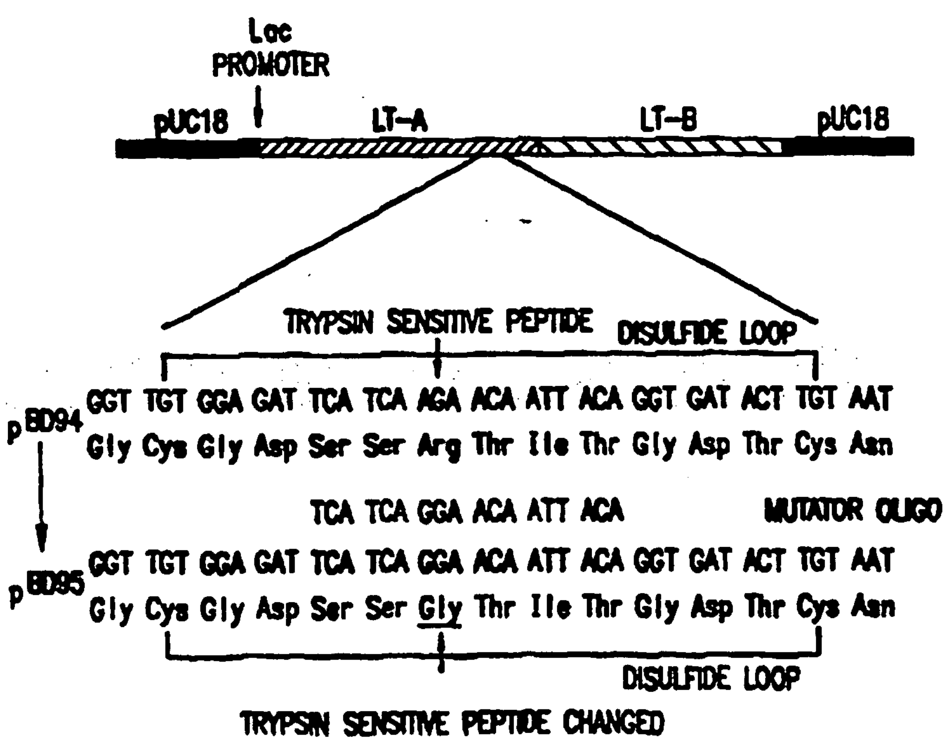

(57) Abstract

Methods and compositions are provided herein for the use f a novel mutant form f E. coli heat-labile enterotoxin which has lost its toxicity but has retained its immunologic activity. This enterotoxin is used in combination with an unrelated antigen to achieve an increased immune response to said antigen when adminsitered as part of an oral vaccine preparation.

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MUTANT ENTEROTOXIN EFFECTIVE AS A NON-TOXIC ORAL ADJUVANT

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1. FIELD OF THE INVENTION

The present invention is directed towards a genetically distinct mutant of E. coli heat-labile enterotoxin (LT) and its use as an oral adjuvant to induce mucosal and serum antibodies. Specifically, the mutant LT is modified by a single amino acid substitution that abolishes its inherent toxicity but leaves intact the adjuvant properties of the molecule.

2. BACKGROUND OF THE INVENTION

Microbial pathogens can infect a host by one of several mechanisms. They may enter through a break in the integument induced by trauma, they may be introduced by vector transmission, or they may interact with a mucosal surface. The majority of human pathogens initiate disease by the last mechanism, i.e., following interaction with mucosal surfaces. Bacterial and viral pathogens that act through this mechanism first make contact with the mucosal surface where they may attach and then colonize, or be taken up by specialized absorptive cells (M cells) in the epithelium that overlay Peyer's patches and other lymphoid follicles [Bockman and Cooper, 1973, Am. J. Anat. 136:455-477; Owen et al., 1986, J. Infect. Dis. 153:1108-1118]. Organisms that enter the lymphoid tissues may be readily killed within the lymphoid follicles, thereby provoking a potentially protective immunological response as antigens are delivered to immune cells within the follicles (e.g., Vibrio cholerae). Alternatively, pathogenic organisms capable of surviving local defense mechanisms may spread from the follicles and subsequently cause local r systemic disease (i.e., Salmonella spp., poliovirus, rotavirus in immunocompr mised hosts).

S cretory IgA (sIgA) antibodies directed against specific virulence determinants of infecting organisms play an important rol in overall mucosal immunity [Cebra et al., 1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harbor Laboratory, New York. p.p. 129-133]. In many cases, it is possible to prevent the initial infection of mucosal surfaces by stimulating production of mucosal sIgA levels directed against relevant virulence determinants of an infecting organism. Secretory IgA may prevent the initial interaction of the pathogen with the mucosal surface by blocking attachment and/or colonization, neutralizing surface acting toxins, or preventing invasion of the host cells. While extensive research has been conducted to determine the role of cell mediated immunity and serum antibody in protection against infectious agents, less is known about the regulation, induction, and secretion of sIgA. Parenterally administered inactivated whole-cell and whole-virus preparations are effective at eliciting protective serum IgG and delayed type hypersensitivity reactions against organisms that have a significant serum phase in their pathogenesis (i.e., Salmonella typhi, Hepatitis B). However, parenteral vaccines are not effective at eliciting mucosal sIgA responses and are ineffective against bacteria that interact with mucosal surfaces and do not invade (e.g., Vibrio cholerae). There is, however, recent evidence that parenterally administered vaccines may be effective against at least one virus, rotavirus, that interacts primarily with mucosal surfaces [Conner et al., 1993, J. Virol. 67:6633-6641]. Protection is presumed to result from transudation of antigen specific IgG onto mucosal surfaces for virus neutralization. Therefore, mechanisms that stimulate both serum and mucosal antibodies are important for effective vaccines.

Oral immunization can be effective for induction of specific sIgA responses if the antigens are presented to th T and B lymphocytes and accessory cells contain d within the Peyer's patches where preferential IgA B-cell d velopment is initiated. The Peyer's patch s contain helper T (TH)-cells

that mediate B-cell isotype switching directly from IgM cells to IgA B-cells. The patches also contain T-cells that initiate terminal B-cell differentiation. The primed B-cells then migrate to the mesenteric lymph nodes and undergo differentiation, enter the thoracic duct, then the general circulation, and subsequently seed all of the secretory tissues of the body, including the lamina propria of the gut and respiratory tract. IgA is then produced by the mature plasma cells, complexed with membrane-bound Secretory Component, and transported onto the mucosal surface where it is available to interact with invading pathogens [Strober and Jacobs, 1985, In: Advances in host defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci (ed.), Raven Press, New York. p.p. 1-30; Tomasi and Plaut, 1985, In: Advances in host defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci (ed.), Raven Press, New York. p.p. 31-61]. The existence of this common mucosal immune system explains in part the potential of live oral vaccines and oral immunization for protection against pathogenic organisms that initiate infection by first interacting with mucosal surfaces.

A number of strategies have been developed for oral immunization, including the use of attenuated mutants of bacteria (i.e., Salmonella spp.) as carriers of heterologous antigens [Cárdenas and Clements, 1992, Clin. Microbiol. Rev. 5:328-342; Clements et al., 1992, In: Recombinant DNA Vaccines: Rationale and Strategy, Isaacson (ed.), Marcel Decker, New York. p.p. 293-321; Clements and Cárdenas, 1990, Res. Microbiol. 141:981-993; Clements and El-Morshidy, 1984, Infect. Immun. 46:564-569], encapsulation of antigens into microspheres composed of poly-DL-lactide-glycolide (PGL), protein-like polymers - proteinoids [Sanitago et al., 1993, Pharmaceutical Research 10:1243-1247], gelatin capsules, different formulations of liposomes [Alving et al., 1986, Vaccine 4:166-172; Garcon and Six, 1993, J. Immunol. 146:3697-3702; Gould-Fogerite and Mannino, 1993, In: Liposome Technol gy 2nd Edition. Vol. III, Gregoriadis (ed.)], adsorption nto nanoparticles, us of lipophilic immune

stimulating complex s (ISCOMS) [Mowat and Donachie, 1991, Immunol gy Today 12:383-385], and addition of bacterial products with known adjuvant properti s [Clements t al., 1988, Vaccine 6:269-277; Elson, 1989, Immunology Today 146:29-33; Lycke and Holmgren, 1986, Immunology 59:301-308; Lycke et al., 1992, Eur. J. Immunol. 22:2277-2281]. The two bacterial products with the greatest potential to function as oral adjuvants are cholera toxin (CT), produced by various strains of V. cholerae, and the heat-labile enterotoxin (LT) produced by some enterotoxigenic strains of Escherichia coli. Although LT and CT have many features in common, these are clearly distinct molecules with biochemical and immunologic differences which make them unique.

The extensive diarrhea of cholera is the result of a potent exo-enterotoxin which causes the activation of adenylate cyclase and a subsequent increase in intracellular levels of cyclic 3'-,5'-adenosine monophosphate (cAMP). Th cholera enterotoxin (CT) is an 84,000 dalton polymeric protein composed of two major, non-covalently associated, immunologically distinct regions or domains ("cholera-A" and "cholera-B") [Finkelstein and LoSpalluto, 1969, J. Exp. M d. 130: 185-202]. Of these, the 56,000 dalton region, or choleragenoid , is responsible for binding of the toxin to the host cell membrane receptor, G_{M1} (galactosyl-N-acetylgalactosaminyl- (sialyl)-galactosyl-glucosyl ceramide), which is found on the surface of essentially all eukaryotic cells. Choleragenoid is composed of five non-covalently associated subunits, while the A region (27,000 daltons) is responsible for the diverse biological effects of the toxin.

The relationship of the two subunits of CT with respect to the immunologic properties of the molecule has been a source of considerable debate. One the one hand, CT is an excellent immunogen that provokes the development of both s rum and mucosal antitoxin antibody r sponses when deliv r d orally. This finding is not new in that cholera pati nts ar known to develop rises in tit rs of antitoxin antibodies

during convalescence from clinical cholera [Finkelstein, 1975, Curr. Top. Microbiol. Immunol. 69: 137-196]. One key finding of those investigating the nature of this response was th observation that CT, unlike most other protein antigens, does not induce oral tolerance against itself [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897; Elson and Ealding, 1984, J. Immunol. 132: 2736-2741]. This was also found to be true when just the B-subunit was fed to mice, an observation substantiated by the cholera vaccine field trials in Bangladesh in which oral immunization with B-subunit combined with killed whole cells gave rise to mucosal as well as systemic antitoxin antibody responses [Svennerholm et al., 1984, J. Infect. Dis. 149: 884-893].

In addition to being a potent oral immunogen, CT has a number of other reported immunologic properties. As indicated above, Elson and Ealding [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897] observed that orally administered CT does not induce tolerance against itself. Moreover, simultaneous oral administration of CT with a soluble protein antigen, keyhole limpet hemocyanin (KLH), resulted in the development of secretory IgA responses against both CT and KLH and also abrogated the induction of oral tolerance against KLH. These findings were subsequently confirmed and extended by Lycke and Holmgren [Lycke and Holmgren, 1986, Immunology 59:301-308]. The confusion arises when one attempts to define the role of the A and B subunits of CT with respect to the adjuvant properties of the molecule. The following observations, as summarized by Elson [Elson, 1989, Immunology Today 146:29-33], are the basis for that confusion:

- CT does not induce oral tolerance against itself [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897].
- CT-B does not induce oral tolerance against itself [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897].
- CT can prevent the induction of tolerance against other antigens with which it is simultaneously deliver d and also serv as an adjuvant for those antigens [Els n and Ealding, 1984, J. Immunol. 133: 2892-2897; Lycke and Holmgren, 1986,

Immunology 59:301-308].

- CT can act as and adjuvant for CT-B [Elson and Ealding, 1984, J. Immunol. 133: 2892-2897].
- Heat aggregated CT has little toxicity but is a potent oral immunogen [Pierce et al., 1983, Infect. Immun. 40: 1112-1118].
- CT-B can serve as an immunologic "carrier" in a traditional hapten-carrier configuration [Cebra et al., 1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harbor Laboratory, New York. p.p. 129-133; McKenzie and Halsey, 1984, J. Immunol. 133: 1818-1824].

A number of researchers have concluded from these findings that the B-subunit must possess some inherent adjuvant activity. The findings of Cebra et al. [Cebra et al., 1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harb r Laboratory, New York. p.p. 129-133], Lycke and Holmgren [Lycke and Holmgren, 1986, Immunology 59:301-308], and Liang et al. [Liang et al., 1988, J. Immunol. 141: 1495-1501] would argue against that conclusion. Cebra et al. [Cebra et al., 1986, In: Vaccines 86, Brown et al. (ed.), Cold Spring Harbor Laboratory, New York. p.p. 129-133] demonstrated that purifi d CT-B was effective at raising the frequency of specific anti-cholera toxin B-cells in Peyer's patches when given intraduodenally but, in contrast to CT, did not result in significant numbers of IgA committed B-cells. Lycke and Holmgren [Lycke and Holmgren, 1986, Immunology 59:301-308] compared CT and CT-B for the ability to enhance the gut mucosal immune response to KLH by measuring immunoglobulin secreting cells in the lamina propria of orally immunized mice. They found no increase in anti-KLH producing cells in response to any dose of B-subunit tested in their system. Finally, Liang et al. [Liang et al., 1988, J. Immunol. 141: 1495-1501] found no adjuvant effect when CT-B was administ red orally in conjunction with inactivated Sendai virus.

Where adjuvant activity has b en obs rved f r isolated B-subunit, it has typically be n for on of two reasons. First, a traditional method of preparing B-subunit has be n to

subject holot xin to dissociation chromatography by g l filtration in the presence of a dissociating agent (i. ., guanidine HCl or formic acid). Th isolated subunits are then pooled and the dissociating agent removed. B-subunit prepared by this technique is invariably contaminated with trace amounts of A-subunit such that upon renaturation a small amount of holotoxin is reconstituted. The second reason has to do with the definition of an immunologic carrier. Like many other soluble proteins, B-subunit can serve as an immunologic vehicle for presentation of antigens to the immune system. If those antigens are sufficiently small as to be poorly immunogenic, they can be made immunogenic in a traditional hapten-carrier configuration. Likewise, there is a "theoretical" immune enhancement associated with B-subunit, especially for oral presentation, in that B-subunit binds to the surface of epithelial cells and may immobilize an attached antigen for processing by the gut associated lymphoid tissues. However, any potential advantage to this mechanism of antigen stabilization may be offset by the distribution of the antigen across non-immunologically relevant tissues, i.e., the surface of intestinal epithelial cells. In context of the mucosal responsiveness, the immunologically relevant sites are the Peyer's patches, especially for antigen-specific T cell-dependent B cell activation [Strober and Jacobs, 1985, In: Advances in host defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci (ed.), Raven Press, New York. p.p. 1-30; Tomasi and Plaut, 1985, In: Advances in host defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci (ed.), Raven Press, New York. p.p. 31-61; Brandtzaeg, 1989, Curr. Top. Microbiol. Immunol. 146: 13-25]. Thus, the events up to isotype switching from IgM cells to IgA B-cells occurs in the Peyer's patches. Antigens localized on the epithelial cell surface <u>may</u> contribute to antigen induced B cell proliferation in that the class II positive villous epithelial cells may act as antigen presenting cells for T c ll activation at the s cr tory site, th reby increasing cytokine production, terminal B cell differentiation, increased expression f

secretory component, and increased external transport of antigen specific IgA [Tomasi, T. B., and A.G. Plaut. 1985, In: Advances in host defense mechanisms. Vol. 4. Mucosal Immunity, Gallin and Fauci (ed.), Raven Press, New York. p.p. 31-61]. The relationships of these events have not been clearly defined for B-subunit as a carrier of other antigens and use of the term "adjuvant" would seem inappropriate for such an effect.

It is clear that the adjuvant property of the molecule resides in the holotoxin in which B-subunit is required for receptor recognition and to facilitate penetration of the A-subunit into the cell. The A-subunit is also required for adjuvant activity, presumably as a function of its ADP-ribosylating enzymatic activity and ability to increase intracellular levels of cAMP (see below). The B-subunit alone may act as a carrier of other antigens in that when conjugated to those antigens they can be immobilized for processing by the gut associated lymphoid tissues.

Although LT and CT have many features in common, these are clearly distinct molecules with biochemical and immunologic differences which make them unique, including a 20% difference in nucleotide and amino acid sequence homology [Dallas and Falkow, 1980, Nature 288: 499-501]. The two toxins have the same subunit number and arrangement, same biological mechanism of action, and the same specific activity in many *in vitro* assays [Clements and Finkelstein, 1979, Infect. Immun. 24:760-769; Clements et al., 1980, Infect. Immun. 24: 91-97].

There are, however, significant differences between these molecules that influence not only their enterotoxic properties, but also their ability to function as adjuvants. To begin with, unlike CT produced by V. cholerae, LT remains cell associated and is only released from E. coli during cell lysis [Clements and Finkelstein, 1979, Infect. Immun. 24:760-769]. CT is secret d from the vibrio as soon as it is synthesized and can be readily identifi d in, and purified fr m, culture supernatants. Consequently, in contrast to CT,

LT is not fully biologically activ wh n first isolat d from the cell. Consistent with the A-B model for bacterial toxins, LT requires proteolysis and disulfide reduction to be fully active. In the absence of proteolytic processing, the enzymatically active A_1 moiety is unable to dissociate from the A_2 component and cannot reach its target substrate (adenylate cyclase) on the basolateral surface of the intestinal epithelial cell. This is also true for CT, but proteases in the culture supernatant, to which the toxin is exposed during purification, perform the proteolysis. Since LT is not fully biologically active, it is difficult to identify during purification using *in vitro* biological assays such as the Y-1 adrenal cell assay or permeability factor assay.

This difference in activation of the isolated material results in differences in response thresholds for LT and CT in biologic systems. For instance, CT induces detectable net fluid secretion in the mouse intestine at a dose of 5-10 μg. LT induces detectable net secretion in the mouse intestine at levels above 100 μg. In the rabbit ligated ileal loop, the difference is dramatic and clear cut. Moreover, in primates LT has been shown not to induce fluid secretion at any dose tested up to 1 milligram. This is 200 times the amount of CT reported to induce positive fluid movement in humans. When LT is exposed to proteolytic enzymes with trypsin-like specificity, the molecule becomes indistinguishable from CT in any biologic assay system. This was demonstrated clearly by Clements and Finkelstein [Clements and Finkelstein, 1979, Infect. Immun. 24:760-769].

In addition to the above reported differences, LT has an unusual affinity for carbohydrate containing matrices. Specifically, LT, with a molecular weight of 90,000, elutes from Sephadex columns (glucose) with an apparent molecular weight of 45,000 and from Agarose columns (galactose) with an apparent molecular weight of 0. That is, it binds to galactose containing matrices and can be lut d from those matrices in pure form by application of galactose. LT binds not only to agarose in columns used f r purification, but m re

importantly, to other biological mol cul s containing galactose, including glycoprot ins and lipopolysaccharides. This l ctin-lik binding prop rty of LT results in a broader receptor distribution on mammalian cells for LT than for CT which binds only to G_{M1}. This may account in part for the reported differences in the abilities of these two molecules to induce different helper T lymphocyte responses [McGhee et al., 1994, Mucosal Immunology Update, Spring 1994, Raven Press, New York. p. 21].

In these studies reported by McGhee et al. [McGhee et al., 1994, Mucosal Immunology Update, Spring 1994, Raven Press, New York. p. 21], it was shown that oral immunization of mice with vaccines such as tetanus toxoid (TT) with CT as a mucosal adjuvant selectively induces T_H2 type cells in Peyer's patches and spleens as manifested by TH cells which produce IL-4 and IL-5, but not IL-2 or INF-gamma. [For a more complete review of the cytokine network see Arai et al., 1990, Ann. Rev. Biochem. 59:783-836] Importantly, when CT was used as a mucosal adjuvant it also enhanced antigen-specific IgE responses in addition to the IgA response. Such enhancement of IgE responses seriously compromises the safety of CT as a mucosal adjuvant due to the prospect of inducing immediate-type hypersensitivity reactions. In contrast, LT induces both T_H1 and T_H2 cells and predominantly antigen-specific IgA responses without IgE responses when used as an orally administered mucosal adjuvant.

The two molecules also have many immunologic differences, as demonstrated by immunodiffusion studies [Clements and Finkelstein, 1978, Infect. Immun. 21: 1036-1039; Clements and Finkelstein, 1978, Infect. Immun. 22: 709-713], *in vitro* neutralization studies, and the partial protection against LT associated E. coli diarrhea in volunteers receiving B-subunit whole cell cholera vaccine [Clemens et al., 1988, J. Infect. Dis. 158: 372-377].

Taken together, these findings demonstrate that LT and CT are unique molecules, despite their apparent similarities, and that LT is a practical oral adjuvant while CT is not.

Th demonstration of the adjuvant properties of LT gr w out of an inv stigation of the influence of LT on the development of tolerance to orally administered antigens by one of the present inventors. It was not clear whether or not LT would also influence the induction of oral tolerance or exhibit the adjuvant effects demonstrated for CT, given the observed differences between the two molecules. Consequently, the present inventors examined a number of parameters, including the effect of LT on oral tolerance to OVA and the role of the two subunits of LT in the observed response, the effect of varying the timing and route of delivery of LT, the effect of prior exposure to OVA on the ability of LT to influence tolerance to OVA, the use of LT as an adjuvant with two unrelated antigens, and the effect of route of immunization on anti-OVA responses. The results obtained from these studies [Clements et al., 1988, Vaccine 6:269-277; Clements et al., 1988, Abstract No. B91, 88th Ann. Meet. Am. Soc. Microbiol.] are summarized below:

1. Simultaneous administration of LT with OVA was shown to prevent the induction of tolerance to OVA and to increase the serum anti-OVA IgG response 30 to 90 fold over OVA primed and PBS primed animals, respectively. This effect was determined to be a function of the enzymatically active A-subunit of the toxin since the B-subunit alone was unable to influence tolerance induction.
2. Animals fed LT with OVA after an initial OVA prime developed a significantly lower serum IgG and mucosal IgA anti-OVA response than those fed LT with OVA in the initial immunization, indicating that prior exposure to the antigen reduces the effectiveness of LT to influence tolerance and its ability to act as an adjuvant. LT was not able to abrogate tolerance once it had been established. This was also found to be true for CT when animals were pre-immunized with OVA prior to oral ovalbumin plus CT and offers some insight into the ben ficial observation that antibody r sponses to nontarg t dietary antigens ar not incr ased when th se adjuvants are used.

3. Serum IgG and mucosal IgA responses in animals r ceiving LT on only a single occasion, that b ing upon first exposure to antigen, were equivalent to responses after three OVA/LT primes, indicating that commitment to responsiveness occurs early and upon first exposure to antigen. It was also demonstrated that the direction of the response to either predominantly serum IgG or mucosal IgA can be controlled by whether or not a parenteral booster dose is administered.

4. Simultaneous administration of LT with two soluble protein antigens results in development of serum and mucosal antibodies against both antigens if the animal has no prior immunologic experience with either. This was an important finding since one possible application of LT as an adjuvant would be for the development of mucosal antibodies against complex antigens, such as killed bacteria or viruses, wher the ability to respond to multiple antigens would be important.

Studies by Tamura et al., [Tamura et al., U.S. Patent No. 5,182,109] demonstrated that LT and/or CT administered intranasally enhanced the antibody titer against a co-administered antigen. However, nowhere in Tamura et al. is it taught that these toxins can induce a protective immune response when administered orally.

Clearly, LT has significant immunoregulatory potential, both as a means of preventing the induction of tolerance to specific antigens and as an adjuvant for orally administered antigens and it elicits the production of both serum IgG <u>and</u> mucosal IgA against antigens with which it is delivered. This raises the possibility of an effective immunization program against a variety of pathogens involving the oral administration of killed or attenuated agents or relevant virulence determinants of specific agents. Howev r, the fact that this "toxin" can stimulate a net lumenal secretory response when proteolytically cleaved, as by gut proteases, or when administered in high enough c nc ntrations orally, may hinder investigation into its potential r pr v nt its use under appropriat conditions. This problem could b

resolved if LT could be "detoxified" without diminishing the adjuvant pr perties of th molecul s. It order to appreciate how this might be accomplished, it is necessary to further analyze the mechanism of action of the LT and CT and the structural and functional relationships of these molecules. As indicated previously, both LT and CT are synthesized as multisubunit toxins with A and B components. After the initial interaction of the toxin with the host cell membrane receptor, the B region facilitates the penetration of the A-subunit through the cell membrane. On thiol reduction, this A component dissociates into two smaller polypeptide chains. One of these, the A_1 piece, catalyzes the ADP-ribosylation of the stimulatory GTP-binding protein (G_S) in the adenylate cyclase enzyme complex on the basolateral surface of the epithelial cell and this results in increasing intracellular levels of cAMP. The resulting increase in cAMP causes secretion of water and electrolytes into the small intestine through interaction with two cAMP-sensitive ion transport mechanisms involving 1) NaCl co-transport across the brush border of villous epithelial cells, and 2) electrogenic Na^+ dependent Cl^- secretion by crypt cells [Field, 1980, In: Secretory diarrhea, Field et al. (ed.), Waverly Press, Baltimore. p.21-30]. The A subunit is also the principal moiety associated with immune enhancement by these toxins. This subunit then becomes a likely target for manipulation in order to dissociate the toxic and immunologic functions of the molecules. A recent report by Lycke et al. [Lycke et al., 1992, Eur. J. Immunol. 22:2277-2281] makes it clear that alterations that affect the ADP-ribosylating enzymatic activity of the toxin and alter the ability to increase intracellular levels of cAMP also prevent the molecule from functioning as an adjuvant. Consequently, another approach to detoxification must be explored.

3. SUMMARY OF THE INVENTION

The present invention is based on the surprising obs rvation that a mutant form of LT, which has lost its t xic eff ct and is devoid of ADP-ribosyltransferase activity, still retains its activity as an immunological adjuvant. The mutant

form f LT differs from the wild-type by a single amino acid substituti n, Arg_{192}-Gly_{192}, rendering a trypsin sensitive site insensitive. The loss of the proteolytic site prevents the proteolytic processing of the A subunit into its toxic form. Native LT is not toxic when first isolated from the bacterium but has the potential to be fully toxic when exposed to proteases such as those found in the mammalian intestine. Th mutant form of LT no longer has the potential to become toxic due to proteolytic activation. This mutant LT (hereinafter mLT) retains the capability of enhancing an animal's immun response (e.g., IgG, IgA) to an antigen unrelated to LT or mLT with no toxic side effects. Experimental evidence shows that mLT has utility as an adjuvant for orally administered antigens; such administration results in the production of serum IgG and/or mucosal sIgA against the antigen with which the mLT is delivered. The present invention provides a method for induction of a serum and/or mucosal immune response in a host to any orally administered antigen which comprises administering to the host an effective amount of mLT in conjunction with oral administration of an effective amount of the antigen. Preferably, the antigen and the mLT are administered initially in a simultaneous dose.

The present method and compositions provide an improved mode of oral immunization for development of serum and mucosal antibodies against pathogenic microorganisms. Production of IgA antibody responses against pathogenic microorganisms which penetrate or invade across mucosal surfaces can be directed to that surface, while a significant serum antibody response can be developed to prevent infection by pathogenic microorganisms against which serum antibody is protective. The present invention is useful for any specific antigen where a specific neutralizing antibody response would be useful in ablating the physiological or disease state associated with that antigen.

The present invention also provid s a composition useful as a component of a vaccin against nterot xic bacterial organisms expr ssing chol ra-like enter toxins and

m thods for its use.

The invention also provides a composition us ful in these methods. The composition comprises an effective amount of mLT in combination with an effective amount of antigen.

4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be understood more fully by reference to the following detailed description of the invention, examples of specific embodiments of the invention and the appended figures in which:

Figure 1. Schematic diagram of the plasmid pBD94, which encodes both subunits A and B under the control of the *lac* promoter. Plasmid pBD95 contains the single base substitution at amino acid residue 192 of subunit A, coding for Gly rather than Arg, which preserves the reading frame but eliminates the proteolytic site. The amino acid sequence corresponding to the region of trypsin sensitivity and the site of the amino acid substitution Arg_{192}-Gly_{192} is shown.

Figure 2. Graphic demonstration of the dose-dependent increase in the levels of ADP-ribosylagmatine as a function of increasing amounts of CT.

Figure 3. Fluid accumulation after feeding 125 μg of native LT but not after feeding 125 μg of mLT to mice. The gut-carcass ratio is defined as the intestinal weight divided by the remaining carcass weight.

Figure 4. Ability of mLT to act as an immunological adjuvant. Figure 4A, Ability of mLT to induce a serum IgG response to OVA. Figure 4B, Ability of mLT to induce a mucosal sIgA response to OVA.

Figure 5. Experimental demonstration that mLT retains the ability to prevent induction of oral tolerance to LT. Figure 5A, Ability of mLT to induce a serum IgG response to LT. Figure 5B, Ability of mLT to induce a mucosal sIgA response to LT.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention encompasses a composition and m thods for its us to promote th production of mucosal and serum antibodies against antig ns that ar simultane usly

orally administer d with a gen tically modified bacterial toxin. The modified toxin is a form of the heat-labile enterotoxin (LT) f E. coli which through g netic engin ering has lost its trypsin sensitive site rendering the molecule non-toxic but yet, unexpectedly, retains its ability to act as an immunological adjuvant. The mutant LT is herein termed "mLT". The invention is based on the discovery that mLT is as effective as LT as an immunological adjuvant, an unexpected and surprising result. mLT no longer has the enzymatic activity of ADP-ribosylation because the A subunit can no longer be proteolytically processed. In contrast to published studies of Lycke and colleagues, which made it clear that alterations that effect the ADP-ribosylating activity of LT also prevent the molecule from functioning as an immunologic adjuvant [Lycke et al., 1992, Eur. J. Immunol. 22:2277-2281], the presently described mLT retains activity as an immunological adjuvant although, as demonstrated in the examples, it does not have ADP-ribosylating activity.

The novel mutant form of the heat-labile enterotoxin of E. coli, mLT, described herein, behaves as an adjuvant and elicits the production of both serum IgG and mucosal sIgA against antigens with which it is delivered. The utility of this surprising discovery is that an adjuvant effective amount of mLT may be utilized in an effective immunization program against a variety of pathogens involving the oral administration of an effective amount of mLT adjuvant in admixture with killed or attenuated pathogens or relevant virulence determinants of specific pathogens with no fear of the real or potential toxic side-effects associated with oral administration of CT or LT.

The present invention supersedes the prior art in that the present invention may be used in a variety of immunological applications where CT, LT, or subunits of CT or LT may have been used, but now with mLT there are no real or potential side-eff cts, such as diarrhea, associated with its us . In contrast to LT, which although not toxic when first isolated from the bacterium, has the potential to be fully

toxic when expos d to prot ases such as those found in the mammalian intestine, mLT does not hav the pot ntial to become toxic due to proteolytic activation.

Another embodiment of the present invention is as a component of a vaccine against enterotoxic organisms which express cholera-like toxins. The present inventors have shown that mLT is not subject to orally induced immune tolerance when administered (see below), therefore mLT can function and is highly desired as a component of vaccines directed against enterotoxic organisms. Current technology provides for vaccines against cholera-like toxin expressing organisms containing killed whole cells and the B subunit of the toxin. By replacing the B subunit with mLT in the vaccine, the vaccine is improved in two different ways. First, mLT, which has both the A and B subunits will now induce an immune response not only to the B subunit but to the A subunit as well. This provides for more epitopes for effective neutralization. Second, the adjuvant activity inherent in mLT will enhance the immune response against the killed whole cell component of the vaccine.

Further, other investigators [Häse et al., 1994, Infect. Immun. 62:3051-3057] have shown that the A subunit, modified so that it is no longer toxic by altering the active site of the ADP-ribosylating enzymatic activity, (as opposed to the proteolytic site which is the subject of the current invention) can induce an immune response against the wild type A subunit. However, the A subunit so modified now lacks immunologic adjuvant activity and is therefore less desirable as a vaccine component than mLT.

Moreover, since antibodies against mLT cross-react with LT and CT, mLT can be used in vaccines directed against many types of enterotoxic bacterial organisms that express cholera-like toxins, such as <u>Escherichia</u> spp. and <u>Vibrio</u> spp.

5.1 PRODUCTION OF mLT

The wild-type LT toxin is encoded on a naturally occurring plasmid found in strains of ent rotoxig nic <u>E. coli</u> capable of producing this toxin. The present inv ntors had

previously cloned the LT gen from a human isolate of E. coli d signat d H10407. This subclone consists of a 5.2 kb DNA fragm nt from th enterotoxin plasmid of H10407 ins rted into the *Pst*I site of plasmid pBR322 [Clements et al, 1983, Infect. Immun. 40:653]. This recombinant plasmid, designated pDF82, has been extensively characterized and expresses LT under control of the native LT promoter. The next step in this process was to place the LT gene under the control of a strong promoter, in this case the *lac* promoter on plasmid pUC18. This was accomplished by isolating the genes for LT-A and LT-B separately and recombining them in a cassette in the vector plasmid. This was an important step because it permitted purification of reasonable quantities of LT and derived mutants for subsequent analysis. This plasmid, designated pBD94, is shown diagrammatically in Figure 1.

Both CT and LT are synthesized with a trypsin sensitive peptide bond that joins the A_1 and A_2 pieces. This peptide bond must be nicked for the molecule to be "toxic". This is also true for diphtheria toxin, the prototypic A-B toxin, and for a variety of other bacterial toxins. If the A_1-A_2 bond is not removed, either by bacterial proteases or intestinal proteases in the lumen of the bowel, the A_1 piece cannot reach its target on the basolateral surface of the intestinal epithelial cell. In contrast to CT, LT is not fully biologically active when first isolated from the cell. LT also requires proteolysis to be fully active and the proteolytic activation does not occur inside of the bacterium. Therefore, one means of altering the toxicity of the molecule without affecting the ADP-ribosylating enzymatic activity would be to remove by genetic manipulation the trypsin sensitive amino acids that join the A_1 and A_2 components of the A subunit. If the molecule cannot be proteolytically cleaved, it will not be toxic. One skilled in the art would predict that the mol cule should, however, retain its ADP-ribosylating enzymatic activity and consequently, its adjuvant function.

Figur 1 shows the sequ nce of the disulfide subt nd d regi n that s parat s the A_1 and A_2 pieces. Within this region

is a singl Arginin residu which is believed to be the site f cleavage n cessary to activat the toxic properties of the mol cule. This region was chang d by site-directed mutagenesis in such a way as to render the molecule insensitive to proteolytic digestion and, consequently, nontoxic.

Site-directed mutagenesis is accomplished by hybridizing to single stranded DNA a synthetic oligonucleotide which is complementary to the single stranded template except for a region of mismatch near then center. It is this region that contains the desired nucleotide change or changes. Following hybridization with the single stranded target DNA, the oligonucleotide is extended with DNA polymerase to create a double stranded structure. The nick is then sealed with DNA ligase and the duplex structure is transformed into an E. coli host. The theoretical yield of mutants using this procedure is 50% due to the semi-conservative mode of DNA replication. In practice, the yield is much lower. There are, however, a number of methods available to improve yield and to select for oligonucleotide directed mutants. The system employed utilized a second mutagenic oligonucleotide to create altered restriction sites in a double mutation strategy.

The next step was to substitute another amino acid for Arg (i.e., GGA = Gly replaces AGA = Arg), thus preserving the reading frame while eliminating the proteolytic site. mLT was then purified by agarose affinity chromatography from one mutant (pBD95) which had been confirmed by sequencing. Alternate methods of purification will be apparent to those skilled in the art. This mutant LT, designated $LT_{(R192G)}$ was then examined by SDS-polyacrylamide gel electrophoresis for modification of the trypsin sensitive bond. Samples were examined with and without exposure to trypsin and compared with native (unmodified) LT. mLT does not dissociate into A_1 and A_2 when incubated with trypsin, thereby indicating that sensitivity to protease has been removed.

5.2 MODE OF ADMINISTRATION OF mLT AND UNRELATED ANTIGENS

In accordance with the present invention, mLT can be administ r d in conjunction with any biol gically rel vant

antigen and/or vaccine, such that an incr ased immune response to said antigen and/or vaccine is achieved. In a preferr d embodiment, the mLT and antigen are administered simultaneously in a pharmaceutical composition comprising an effective amount of mLT and an effective amount of antigen. The mode of administration is oral. The respective amounts of mLT and antigen will vary depending upon the identity of the antigen employed and the species of animal to be immunized. In one embodiment, the initial administration of mLT and antigen is followed by a boost of the relevant antigen. In another embodiment no boost is given. The timing of boosting may vary, depending on the antigen and the species being treated. The modifications in dosage range and timing of boosting for any given species and antigen are readily determinable by routine experimentation. The boost may be of antigen alone or in combination with mLT. The mode of administration of the boost may either be oral, nasal, or parenteral; however, if mLT is used in the boost, the administration is preferably oral.

The methods and compositions of the present invention are intended for use both in immature and mature vertebrat s, in particular birds, mammals, and humans. Useful antigens, as examples and not by way of limitation, would include antigens from pathogenic strains of bacteria (Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrheae, Neisseria meningitidis, Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Clostridium tetani, Hemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenae, Klebsiella rhinoscleromotis, Staphylococcus aureus, Vibrio colerae, Escherichia coli, Pseudomonas aeruginosa, Campylobacter (Vibrio) fetus, Aeromonas hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Treponema pallidum, Treponema pertenue, Treponema caraten um, Borrelia vincentii, Borrelia burgdorferi, Leptospira icterohemorrhagia , Mycobact rium tuberculosis, Toxoplasma

gondii, Pneumocystis carinii, Francisella tularensis, Brucella abortus, Brucella suis, Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki, Rickettsia tsutsugumushi, Chlamydia spp.); pathogenic fungi (Coccidioides immitis, Aspergillus fumigatus, Candida albicans, Blastomyces dermatitidis, Cryptococcus neoformans, Histoplasma capsulatum); protozoa (Entomoeba histolytica, Trichomonas tenas, Trichomonas hominis, Trichomonas vaginalis, Trypanosoma gambiense, Trypanosoma rhodesiense, Trypanosoma cruzi, Leishmania donovani, Leishmania tropica, Leishmania braziliensis, Pneumocystis pneumonia, Plasmodium vivax, Plasmodium falciparum, Plasmodium malaria); or Helminiths (Enterobius vermicularis, Trichuris trichiura, Ascaris lumbricoides, Trichinella spiralis, Strongyloides stercoralis, Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium, and hookworms) either presented to the immune system in whole cell form or in part isolated from media cultures designed to grow said organisms which are well know in the art, or protective antigens from said organisms obtained by genetic engineering techniques or by chemical synthesis.

Other relevant antigens would be pathogenic viruses (as examples and not by limitation: Poxviridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2, Adenoviridae, Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae, influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non-A/Non-B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus) either presented to the immune system in whole or in part isolated from media cultures designed to grow such viruses which are well known in the art or protective antigens therefrom obtained by genetic engineering techniques or by chemical synthesis.

Further exampl s of rel vant antigens includ , but are n t limited to, vaccines. Examples of such vaccin s include,

but are not limited to, influenza vaccin , p rtussis vaccine, diphtheria and tetanus toxoid combined with pertussis vaccine, hepatitis A vaccine, hepatitis B vaccine, hepatitis C vaccine, hepatitis E vaccine, Japanese encephalitis vaccine, herpes vaccine, measles vaccine, rubella vaccine, mumps vaccine, mixed vaccine of measles, mumps and rubella, papillomavirus vaccine, parvovirus vaccine, respiratory syncytial virus vaccine, Lyme disease vaccine, polio vaccine, malaria vaccine, varicella vaccine, gonorrhea vaccine, HIV vaccine, schistosomiasis vaccine, rota vaccine, mycoplasma vaccine pneumococcal vaccine, meningococcal vaccine and others. These can be produced by known common processes. In general, such vaccines comprise either the entire organism or virus grown and isolated by techniques well known to the skilled artisan or comprise relevant antigens of these organisms or viruses which are produced by genetic engineering techniques or chemical synthesis. Their production is illustrated by, but not limited to, as follows:

Influenza vaccine: a vaccine comprising the whole or part of hemagglutinin, neuraminidase, nucleoprotein and matrix protein which are obtainable by purifying a virus, which is grown in embryonated eggs, with ether and detergent, or by genetic engineering techniques or chemical synthesis.

Pertussis vaccine: a vaccine comprising the whole or a part of pertussis toxin, hemagglutinin and K-agglutin which are obtained from avirulent toxin with formalin which is extracted by salting-out or ultracentrifugation from the culture broth or bacterial cells of Bordetella pertussis, or by genetic engineering techniques or chemical synthesis.

Diphtheria and tetanus toxoid combined with pertussis vaccine: a vaccine mixed with pertussis vaccine, diphtheria and tetanus toxoid.

Japanese encephalitis vaccine: a vaccine comprising the whole or part of an antigenic protein which is obtained by culturing a virus intracerebrally in mic and purifying the virus particles by centrifugation or ethyl alcohol and inactivating th same, r by genetic ngin ring techniques or

chemical synthesis.

Hepatitis B vaccin : a vaccin comprising the whol or part of an antigen protein which is btained by isolating and purifying the HBs antigen by salting-out or ultracentrifugation, obtained from hepatitis carrying blood, or by genetic engineering techniques or by chemical synthesis.

Measles vaccine: a vaccine comprising the whole or part of a virus grown in a cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering or chemical synthesis.

Rubella vaccine: a vaccine comprising the whole or part of a virus grown in cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mumps vaccine: a vaccine comprising the whole or part of a virus grown in cultured rabbit cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mixed vaccine of measles, rubella and mumps: a vaccine produced by mixing measles, rubella and mumps vaccines.

Rota vaccine: a vaccine comprising the whole or part of a virus grown in cultured MA 104 cells or isolated from the patient's feces, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mycoplasma vaccine: a vaccine comprising the whole or part of mycoplasma cells grown in a liquid culture medium for mycoplasma or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Those conditions for which effective prevention may be achieved by the present method will be obvious to the skilled artisan.

The vaccine preparation compositions of the present invention can be prepared by mixing the above illustrated antigens and/or vaccines with mLT at a desired ratio. The preparation should be conducted strictly as ptically, and ach c mponent should also be aseptic. Pyrog ns r allergens sh uld naturally be removed as completely as possible. The

antig n preparation of th present inventi n can be used by preparing th antigen *per s* and the mLT separately.

Further, the pres nt inv ntion ncompass s a kit comprising an effective amount of antigen and an adjuvant effective amount of mLT. In use, the components of the kit can either first be mixed together and then administered orally or the components can be administered orally separately within a short time of each other.

The vaccine preparation compositions of the present invention can be combined with either a liquid or solid pharmaceutical carrier, and the compositions can be in the form of tablets, capsules, powders, granules, suspensions or solutions. The compositions can also contain suitable preservatives, coloring and flavoring agents, or agents that produce slow release. Potential carriers that can be used in the preparation of the pharmaceutical compositions of this invention include, but are not limited to, gelatin capsules, sugars, cellulose derivations such as sodium carboxymethyl cellulose, gelatin, talc, magnesium stearate, vegetable oil such as peanut oil, etc., glycerin, sorbitol, agar and wat r. Carriers may also serve as a binder to facilitate tabletting of the compositions for convenient oral administration.

The vaccine preparation composition of this invention may be maintained in a stable storage form for ready use by lyophilization or by other means well known to those skill d in the art. For oral administration, the vaccine preparation may be reconstituted as a suspension in buffered saline, milk, or any other physiologically compatible liquid medium. Th medium may be made more palatable by the addition of suitable coloring and flavoring agents as desired.

Administration of the vaccine preparation compositions may be preceded by an oral dosage of an effective amount of a gastric acid neutralizing agent. While many compounds could be used for this purpose, sodium bicarbonate is preferred. Alternatively, th vaccin compositions may be delivered in enteric coated capsules (i.e., capsules that dissolve only aft r passing through th stomach).

6. EXAMPLES

The following xamples ar presented for purposes of illustration only and are not intended to limit th scop of the invention in any way.

6.1 CONSTRUCTION OF mLT

The wild-type LT toxin is encoded on a naturally occurring plasmid found in strains of enterotoxigenic E. coli capable of producing this toxin. The present inventors had previously cloned the LT gene from a human isolate of E. coli designated H10407. This subclone consists of a 5.2 kb DNA fragment from the enterotoxin plasmid of H10407 inserted into the *Pst*I site of plasmid pBR322 [Clements et al., 1983, Infect. Immun. 40:653]. This recombinant plasmid, designated pDF82, has been extensively characterized and expresses LT under control of the native LT promoter. The next step in this process was to place the LT gene under the control of a strong promoter, in this case the *lac* promoter on plasmid pUC18. This was accomplished by isolating the genes for LT-A and LT-B separately and recombining them in a cassette in the vector plasmid. This was an important step because it permitted purification of reasonable quantities of LT and derived mutants for subsequent analysis. This plasmid, designated pDF94, is shown diagrammatically in Figure 1.

Both CT and LT are synthesized with a trypsin sensitive peptide bond that joins the A_1 and A_2 pieces. This peptide bond must be nicked for the molecule to be "toxic". This is also true for diphtheria toxin, the prototypic A-B toxin, and for a variety of other bacterial toxins. If the A_1-A_2 bond is not removed, either by bacterial proteases or intestinal proteases in the lumen of the bowel, i.e., proteolytic processing or activation, the A_1 piece cannot reach its target on the basolateral surface of the intestinal epithelial cell. In contrast to CT, LT is not fully biologically active when first isolated from the cell. LT also requir s proteolysis to be fully active and the proteolytic activation do s not occur inside of the bacterium. Therefor , one means of altering the toxicity of the molecule without

aff cting the ADP-ribosylating enzymatic activity would be to remove by genetic manipulation the trypsin sensitive amino acids that join the A_1 and A_2 components of the A subunit. If the molecule cannot be proteolytically cleaved, it will not be toxic. One skilled in the art would predict that the molecule should, however, retain its ADP-ribosylating enzymatic activity and consequently, its adjuvant function.

Figure 1 shows the sequence of the disulfide subtended region that separates the A_1 and A_2 pieces. Within this region is a single Arginine residue which is believed to be the site of cleavage necessary to activate the toxic properties of the molecule. This region was changed by site-directed mutagenesis is such a way as to render the molecule insensitive to proteolytic digestion and, consequently, nontoxic.

Site-directed mutagenesis is accomplished by hybridizing to single stranded DNA a synthetic oligonucleotide which is complementary to the single stranded template except for a region of mismatch near then center. It is this region that contains the desired nucleotide change or changes. Following hybridization with the single stranded target DNA, the oligonucleotide is extended with DNA polymerase to cr at a double stranded structure. The nick is then sealed with DNA ligase and the duplex structure is transformed into an E. coli host. The theoretical yield of mutants using this procedure is 50% due to the semi-conservative mode of DNA replication. In practice, the yield is much lower. There are, however, a number of methods available to improve yield and to select for oligonucleotide directed mutants. The system employed utilized a second mutagenic oligonucleotide to create altered restriction sites in a double mutation strategy.

The next step was to substitute another amino acid for Arg (i.e., GGA = Gly replaces AGA = Arg), thus preserving the reading frame while eliminating the proteolytic site. mLT was then purified by agarose affinity chromatography from one mutant (pBD95) which had b n confirm d by sequencing. Alternate methods of purification will be apparent to thos skilled in th art. This mutant LT, designated $LT_{(R192G)}$ was

then examin d by SDS-polyacrylamide gel electrophoresis for modification of the trypsin s nsitive bond. Samples were examined with and without exposure to trypsin and compared with native (unmodified) LT. mLT does not dissociate into A_1 and A_2 when incubated with trypsin, thereby indicating that sensitivity to protease has been removed.

6.2 EFFECT OF mLT ON Y-1 ADRENAL CELLS

It would be predicted by one skilled in the art that mLT would not be active in the Y-1 adrenal cell assay. This prediction would be based upon previous findings [Clements and Finkelstein, 1979, Infect. Immun. 24:760-769] that un-nicked LT was more than 1,000 fold less active in this assay system than was CT and that trypsin treatment activated LT to the same level of biological activity as CT in this assay. It was presumed that the residual activity of LT observed in this assay in the absence of trypsin activation was a function of some residual protease activity which could not be accounted for. For instance, trypsin is used in the process of subculturing Y-1 adrenal cells. It was therefore assumed that LT that could not be nicked would be completely inactive in the Y-1 adrenal cell assay. Results are shown in Table I.

TABLE I

Toxin	Trypsin Activated	Specific Activity [a]
Cholera Toxin	-	15
LT	-	60
LT	+	15
$LT_{(R192G)}$	-	48,800
$LT_{(R192G)}$	+	48,800

[a]Minimum dose (picograms per well) required to produce (>50%) cell rounding.

Table I demonstrates the unexp ct d finding that mLT

r tained a basal lev l of activity in the Y-1 adrenal cell assay even th ugh it could not be proteolytically proc ssed. As shown in Table I, CT and native LT tr ated with trypsin have the same level of activity (15 pg) on Y-1 adrenal cells. By contrast, mLT (48,000 pg) was >1,000 fold less active than CT or native LT and could not be activated by trypsin. Th residual basal activity undoubtedly reflects a different and here-to-fore unknown pathway of adrenal cell activation than that requiring separation of the A_1 - A_2 linkage.

6.3 ADP-RIBOSYLATING ENZYMATIC ACTIVITY OF mLT

Because the mutation replacing Arg_{192} with Gly_{192} does not alter the enzymatic site of the A_1 moiety, one skilled in the art would predict that mLT would retain its ADP-ribosylating enzymatic activity. To examine this property, the NAD-Agmatine ADP-ribosyltransferase Assay was employed [Moss et al., 1993, J. Biol. Chem. 268:6383-6387]. As shown in Figure 2, CT produces a dose-dependent increase in the levels of ADP-ribosylagmatine, a function of the ADP-ribosyltransferase activity of this molecule.

TABLE II

ADP-Ribosyltransferase Activity of CT, native LT, and $LT_{(R192G)}$					
Experiment	1	2	3	4	Mean±SEM
No Toxin	ND	9.12	5.63	14.17	9.64±2.48
1μgCT	ND	17.81	17.60	25.75	20.39±2.68
10μgCT	ND	107.32	111.28	104.04	107.55±2.09
100μgCT	351.55	361.73	308.09	ND	340.46±16.45
100μgLT	17.32	14.48	13.86	ND	15.22±1.07
100μgLT +Trypsin	164.10	189.89	152.96	ND	168.98±10.94
100μg $LT_{(R192G)}$	14.58	12.34	9.30	ND	12.07±1.53
100μg $LT_{(R192G)}$ +Trypsin	14.73	8.90	10.47	ND	11.37±1.74
ND= Not Done data expressed in fMoles min^{-1}					

Table II demonstrates in tabular form the unexpected finding that mLT lacked any detectable ADP-ribosylating enzymatic activity, with or without trypsin activation, even though the enzymatic site had not been altered and there was a demonstratable basal level of activity in the Y-1 adrenal cell assay.

6.4 ENTEROTOXIC ACTIVITY OF mLT

Because of the unexpected finding that mLT lacks any detectable ADP-ribosylating enzymatic activity, with or without trypsin activation, even though the enzymatic site has not been altered and the additional finding that there is a basal level of activity in the Y-1 adrenal cell assay, it was unclear whether mLT would retain any of its enterotoxic properties. An ideal adjuvant formulation of mLT would retain its ability to act as an immunological adjuvant but would lack th real r potential side-effects, such as diarrh a,

associat d with the use of LT or CT. Figure 3 demonstrates that mLT does not induce net fluid secretion in the patent mouse model, ev n at a dose of 125 μg. This dos is more than five times the adjuvant effective dose for LT in this model. Importantly, the potential toxicity of native LT can be seen at this level.

6.5 ADJUVANT ACTIVITY OF mLT

One skilled in the art would predict that since mLT possessed no demonstrable ADP-ribosyltransferase activity and is not enterotoxic, it would lack adjuvant activity. This prediction would be based upon the report by Lycke et al. [Lycke et al., 1992, Eur. J. Immunol. 22:2277-2281] where it is made clear that alterations that affect the ADP-ribosylating enzymatic activity of the toxin and alter the ability to increase intracellular levels of cAMP also prevent the molecule from functioning as an adjuvant. As demonstrated above, mLT has no ADP-ribosylating enzymatic activity and only some undefined basal activity in Y-1 adrenal cells, and induces no net fluid secretion in the patent mouse model.

In order to examine the adjuvant activity of mLT th following experiment was performed. Three groups of BALB/c mice were immunized. Animals were inoculated intragastrically with a blunt tipped feeding needle (Popper & Sons, Inc., New Hyde Park, New York). On day 0, each group was immunized orally as follows: Group A received 0.5 ml of PBS containing 5 mg of OVA, Group B received 0.5 ml of PBS containing 5 mg of OVA and 25 μg of native LT, and Group C received 0.5 ml of PBS containing 5 mg of OVA and 25 μg of mLT. Each regimen was administered again on days 7 and 14. On day 21, all animals were boosted i.p. with 1 μg of OVA in 20% Maalox. One week after the i.p. inoculation animals were sacrificed and assayed for serum IgG and mucosal IgA antibodies directed against OVA and LT by ELISA.

Reagents and antisera for the ELISA were obtained from Sigma Chemical Co. Samples for ELISA were serially dilut d in phosphat buffered saline (pH 7.2)-0.05% Tween 20 (PBS-TWEEN). For anti-LT determinations, microtiter plat s were precoated

with 1.5 μg per well of mixed gangliosides (Type III), then with 1 μg per well of purified LT. Anti-OVA was determined on microtiter plates precoated with 10 μg per well of OVA. Serum anti-LT and anti-OVA were determined with rabbit antiserum against mouse IgG conjugated to alkaline phosphatase. Mucosal anti-LT and anti-OVA IgA were assayed with goat antiserum against mouse IgA [alpha-chain specific] followed by rabbit antiserum against goat IgG conjugated to alkaline phosphatase. Reactions were stopped with 3N NaOH. Values for IgG and IgA were determined from a standard curve with purified mouse myeloma proteins (MOPC 315, gA(IgA12); MOPC 21, gG1: Litton Bionetics, Inc., Charleston, SC).

6.5.1 SERUM IgG ANTI-OVA

As shown in the Figure 4A, animals primed orally with OVA and LT developed a significantly higher serum IgG anti-OVA response following subsequent parenteral immunization with OVA (4,058 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-OVA response) (Student t-test p= .031). Significantly, animals primed orally with OVA and mLT also developed a significantly higher serum IgG anti-OVA response following subsequent parenteral immunization with OVA (1,338 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-OVA response) (Student t-test p= .0007).

6.5.2 MUCOSAL sIgA ANTI-OVA

As shown in the Figure 4B, similar results were obtained when anti-OVA IgA responses were compared within these same groups of animals. Animals primed orally with OVA and LT developed a significantly higher mucosal IgA anti-OVA response following subsequent parenteral immunization with OVA (869 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-OVA response) (Student t-test p= .0131). As above, animals primed orally with OVA and mLT also developed a significantly high r mucosal IgA anti-OVA response following subsequent parenteral immunizati n with OVA (230 ng/ml) than those primed with OVA

alon and subsequ ntly immunized parenterally with OVA (No detectable anti-OVA response) (Student t-t st p= .0189).

6.5.3 <u>SERUM IgG ANTI-LT</u>

The ability of LT and mLT to elicit an anti-LT antibody response in these same animals was also examined. This was important in that it would provide an indication of whether the mutant LT was able to prevent induction of tolerance to itself in addition to functioning as an adjuvant for other proteins. As shown in Figure 5A, animals primed orally with OVA <u>and</u> LT developed a significantly higher serum IgG anti-LT response following subsequent parenteral immunization with OVA (342 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-LT response) (Student t-test p= .0005). Animals primed orally with OVA <u>and</u> mLT also developed a significantly higher serum IgG anti-LT response following subsequent parenteral immunization with OVA (552 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-LT response) (Student t-test p= .0026).

6.5.4 <u>MUCOSAL sIgA ANTI-LT</u>

As shown in the Figure 5B, similar results were obtained when anti-LT IgA responses were compared within th se same groups of animals. Animals primed orally with OVA <u>and</u> LT developed a significantly higher mucosal IgA anti-LT response following subsequent parenteral immunization with OVA (4,328 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-LT response) (Student t-test p= .0047). As above, animals primed orally with OVA <u>and</u> mLT also developed a significantly higher mucosal IgA anti-LT response following subsequent parenteral immunization with OVA (1,463 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (No detectable anti-LT response) (Student t-test p= .0323).

7. <u>DEPOSIT OF MICROORGANISMS</u>

The following plasmid was deposited with th Am rican Typ Cultur C llection (ATCC), Rockville, MD on August 18,

1994, and has been assigned the indicated accession number:

Plasmid	Accession Number
pBD95 in *E. coli* LTR192G	ATCC 69683

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

It is also to be understood that all base pair and amino acid residue numbers and sizes given for nucleotides and peptides are approximate and are used for purposes of description.

A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on pages 32-33, lines 35-37 and 1-3 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit August 18, 1994 Accession Number 69683

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

Elnora Rivera, Paralegal Specialist
Receiving Office PCT
(703) 305-3165
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS:

1. A composition comprising a mutant form of E. coli heat-labile enterotoxin holotoxin which has immunologic adjuvant activity but lacks ADP-ribosylating enzymatic activity as measured by the NAD-Agmatine ADP-ribosyltransferase Assay.

2. A composition comprising a mutant form of E. coli heat-labile enterotoxin holotoxin which has immunologic adjuvant activity, in which the A subunit of the holotoxin is not cleaved by trypsin.

3. The composition of claim 1 or 2 which is encoded by plasmid PBD95 contained in E. coli LTR192G having the ATCC accession number 69683, which expresses both subunit A and subunit B of the E. coli heat-labile enterotoxin.

4. A vaccine preparation comprising an antigen in combination with the composition according to claim 1 or 2.

5. The vaccine preparation of claim 4 in which the antigen is selected from the group consisting of influenza vaccine, varicella vaccine, diphtheria toxoid, tetanus toxoid, pertussis vaccine, Japanese encephalitis vaccine, mixed vaccine of pertussis, diphtheria and tetanus toxoid, Lyme disease vaccine, polio vaccine, malaria vaccine, herpes vaccine, HIV vaccine, papillomavirus vaccine, hepatitis B vaccine, rota vaccine, Campylobacter vaccine, cholera vaccine, enteropathogenic E. coli vaccine, enterotoxic E. coli vaccine, schistosomiasis vaccine, measles vaccine, rubella vaccine, mumps vaccine, combined vaccine of measles, rubella and mumps, and mycoplasma vaccine.

6. A compositi n useful in producing a protectiv immune r sponse to a pathogen in a host comprising an admixture f an effective amount of an antigen and an adjuvant

effective amount of the composition according to claim 1 or 2.

7. A kit useful in producing a protective immune response in a host to a pathogen comprising two components; (a) an effective amount of antigen and (b) an adjuvant effective amount of a mutant E. coli heat-labile enterotoxin holotoxin which has immunologic adjuvant activity but lacks ADP-ribosylating enzymatic activity as measured by the NAD-Agmatine ADP-ribosyltransferase Assay, wherein both said components are in an orally acceptable carrier and said components may be administered either after having been mixed together or separately within a short time of each other.

8. The composition of claim 1 or 6 in which the A subunit is not cleaved by trypsin.

9. The composition of claim 4 in which the A subunit is not cleaved by trypsin.

10. The kit of claim 7 in which the A subunit is not cleaved by trypsin.

11. The composition of claim 1 or 6 in which amino acid Arg_{192} of the wild type E. coli heat-labile enterotoxin is replaced by Gly_{192}.

12. The composition of claim 4 in which amino acid Arg_{192} of the wild-type E. coli heat-labile enterotoxin is replaced by Gly_{192}.

13. The kit of claim 7 in which amino acid Arg_{192} of the wild-type E. coli heat-labile enterotoxin is replaced by Gly_{192}.

14. A m thod of creating or sustaining a protective or adaptive immune response to an antigen in a host comprising

orally administering an admixture of an effective amount of the antig n and an adjuvant effective amount of a mutant E. coli h at-labile enterotoxin holotoxin which has immunologic adjuvant activity but lacks ADP ribosylating enzymatic activity as measured by the NAD-Agmatine ADP-ribosyltransferase Assay in an orally acceptable pharmaceutical carrier.

15. The method of claim 14 where a serum response is produced.

16. The method of claim 14 where a mucosal response is produced.

17. The method of claim 14 wherein a subsequent boost of antigen is provided.

18. The method of claim 14 wherein the antigen is derived from the group consisting of bacteria, viruses, protozoa, fungi, helminths and other microbial pathogens.

19. The method of claim 14 wherein the admixture is delivered in a single dose.

20. A method of inducing a protective immune response against an enterotoxic bacterial organism comprising using mLT as a component of a vaccine directed against the enterotoxic bacterial organism.

21. The method of claim 20 wherein the enterotoxic bacterial organism is selected from the group consisting of enterotoxic bacterial organisms which express cholera-like toxins.

22. The method of claim 20 wherein the enterotoxic bacterial organism is select d from the group consisting of Escherichia spp. and Vibrio spp.

AMENDED CLAIMS

[received by the International Bureau on 12 January 1996 (12.01.96); original claims 1-22 replaced by new claims 1-25 (4 pages)]

1. A composition comprising a mutant form f a bacterial enterotoxin hol toxin which has reduced toxicity at an adjuvant effective dose, but retains immunologic adjuvant activity.

2. The composition of claim 1, which is rendered insensitive to proteolytic activation.

3. The composition of claim 1, which is a mutant form of E. coli heat-labile enterotoxin holotoxin which is distinct from native LT in that is has immunologic adjuvant activity but reduced ADP-ribosylating activity.

4. The composition of claim 1, which is a mutant form of E. coli heat-labile enterotoxin holotoxin which is distinct from native LT in that the A subunit of the holotoxin is rendered insensitive to proteolytic activation.

5. The composition of claim 1 which is encoded by the plasmid pBD95 contained in E. coli LTR192G having the ATCC accession number 69683, which expresses both subunit A and subunit B of the E. coli heat-labile enterotoxin.

6. A vaccine preparation comprising an antigen in combination with the composition according to claim 1.

7. An oral vaccine preparation comprising an antigen in combination with the composition according to claim 1.

8. The vaccine preparation of claim 6 or 7, in which the antigen is a bacterial antigen of pathogenic bacteria sel cted from th group consisting of Streptococcus spp., Neisseria spp., Corynebacterium spp., Clostridium spp., Hemophilus spp., Klebsiella spp., Staphylococcus spp., Vibrio

spp., Escherichia spp., Pseudomonas spp., Campylobacter spp., Aeromonas spp., Bacillus spp., Edwardsiella spp., Yersinia spp., Shig lla spp., Salmonella spp., Trepon ma spp., Borrelia spp., Leptospira spp., Mycobacterium spp., Toxoplasma spp., Pneumocystis spp., Francisella spp., Brucella spp., Mycoplasma spp., Rickettsia spp. and Chlamydia spp.

9. The vaccine preparation of claim 6 or 7, in which the antigen is selected from the group consisting of influenza vaccine, varicella vaccine, diphtheria toxoid, tetanus toxoid, pertussis vaccine, Japanese encephalitis vaccine, mixed vaccine of pertussis, diphtheria and tetanus toxoid, Lyme disease vaccine, polio vaccine, malaria vaccine, herpes vaccine, HIV vaccine, papillomavirus vaccine, hepatitis B vaccine, rotavirus vaccine, Campylobacter vaccine, cholera vaccine, enteropathogenic E. coli vaccine, enterotoxic E. coli vaccine, Salmonella vaccine, Shigella vaccine, schistosomiasis vaccine, measles vaccine, rubella vaccine, mumps vaccine, combined vaccine of measles, rubella and mumps, and mycoplasma vaccine.

10. A composition useful in producing a protective immune response to a pathogen in a host comprising an admixture of an effective amount of an antigen and an adjuvant effective amount of the composition according to claim 1.

11. An oral composition useful in producing a protective immune response to a pathogen in a host comprising an admixture of an effective amount of an antigen and an adjuvant effective amount of the composition according to claim 1.

12. A mutant enterotoxin as claimed in claim 1 for use in medicine.

13. A kit useful in producing a prot ctiv immune r sponse in a host to a pathogen comprising two components;

(a) an ffective amount of antig n and (b) an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin h lotoxin which is distinct from nativ LT and CT in that the A subunit of th holotoxin has been rendered insensitive to proteolytic activation, but the holotoxin retains immunologic adjuvant activity, wherein both said components are in an orally acceptable carrier and said components may be administered either after having been mixed together or separately within a short time of each other.

14. The composition of claim 1 or 10, in which amino acid Arg_{192} of the wild type *E. coli* heat-labile enterotoxin is replaced by Gly_{192}.

15. The vaccine preparation of claim 6 or 7, in which amino acid Arg_{192} of the wild-type *E. coli* heat-labile enterotoxin is replaced by Gly_{192}.

16. The kit of claim 13, in which amino acid Arg_{192} of the wild-type *E. coli* heat-labile enterotoxin is replaced by Gly_{192}.

17. A method of creating or sustaining a protective or adaptive immune response to an antigen in a host comprising orally administering an admixture of an effective amount of the antigen and an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin which is distinct from native LT and CT in that the A subunit of the holotoxin has been rendered insensitive to proteolytic activation, but the holotoxin retains immunologic adjuvant activity in an orally acceptable pharmaceutical carrier.

18. The method of claim 17, where a serum response is produced.

19. The method f claim 17, where a mucosal response is produc d.

20. The method of claim 17, wherein a subs quent boost of antigen is provid d.

21. The method of claim 17, wherein the antigen is derived from the group consisting of bacteria, viruses, protozoa, fungi, helminths and other microbial pathogens.

22. The method of claim 17, wherein the admixture is delivered in a single dose.

23. A method of inducing a protective immune response against an enterotoxic bacterial organism comprising using mLT as a component of a vaccine directed against the enterotoxic bacterial organism.

24. The method of claim 23, wherein the enterotoxic bacterial organism is selected from the group consisting of enterotoxic bacterial organisms which express cholera-like toxins.

25. The method of claim 24, wherein the enterotoxic bacterial organism is selected from the group consisting of Escherichia spp. and Vibrio spp.

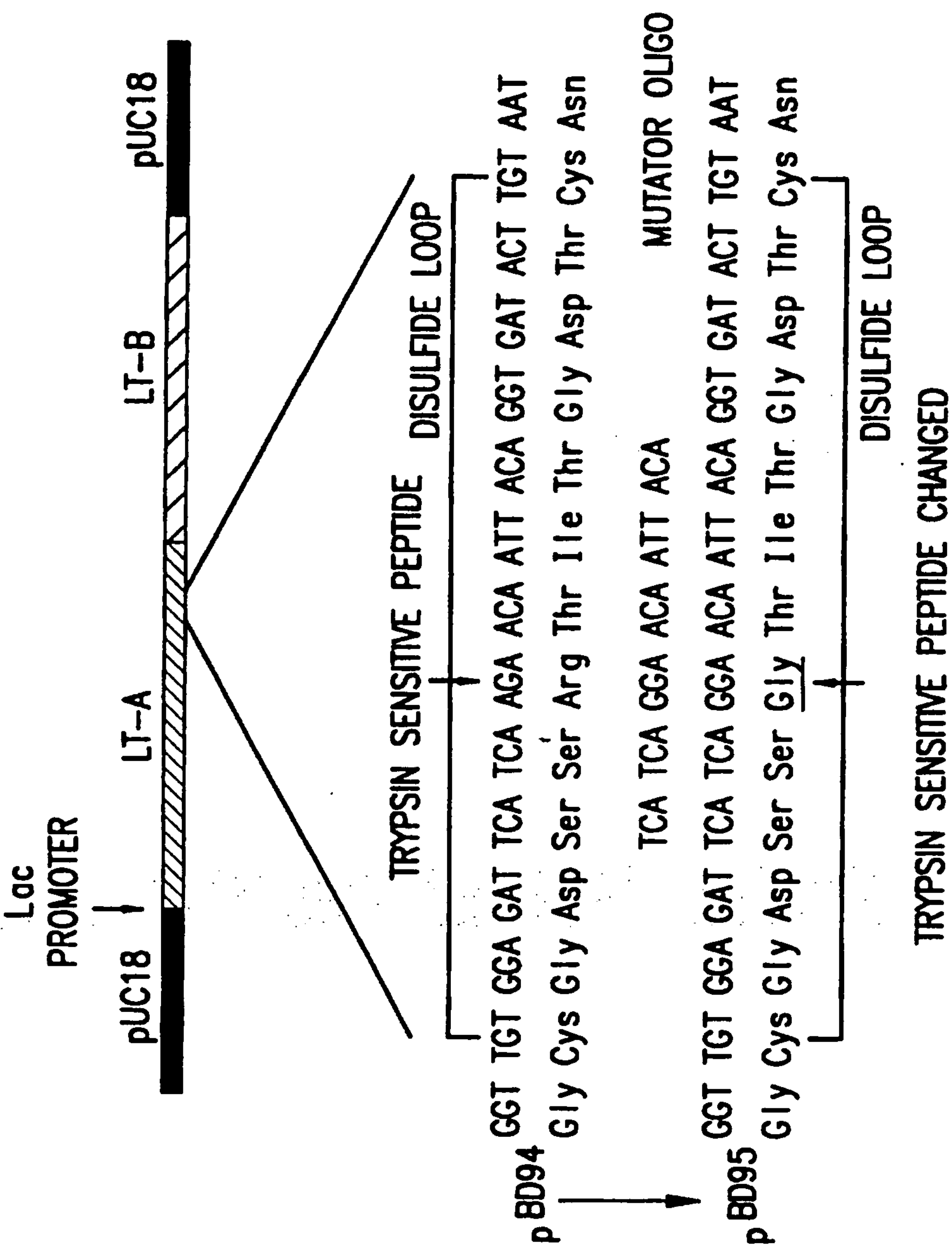
pUC18
Lac PROMOTER
LT-A
LT-B
pUC18
TRYPSIN SENSITIVE PEPTIDE
DISULFIDE LOOP
pBD94
GGT TGT GGA GAT TCA TCA AGA ACA ATT ACA GGT GAT ACT TGT AAT
Gly Cys Gly Asp Ser Ser Arg Thr Ile Thr Gly Asp Thr Cys Asn
TCA TCA GGA ACA ATT ACA
MUTATOR OLIGO
pBD95
GGT TGT GGA GAT TCA TCA GGA ACA ATT ACA GGT GAT ACT TGT AAT
Gly Cys Gly Asp Ser Ser Gly Thr Ile Thr Gly Asp Thr Cys Asn
DISULFIDE LOOP
TRYPSIN SENSITIVE PEPTIDE CHANGED

FIG.1

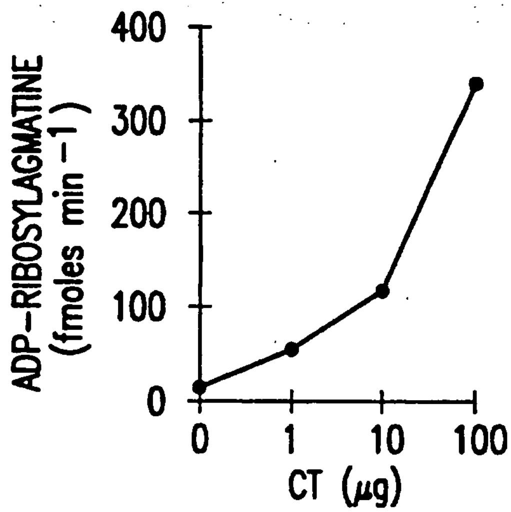
400
300
200
100
0
ADP–RIBOSYLAGMATINE (fmoles min −1)
0
1
10
100
CT (μg)

FIG.2

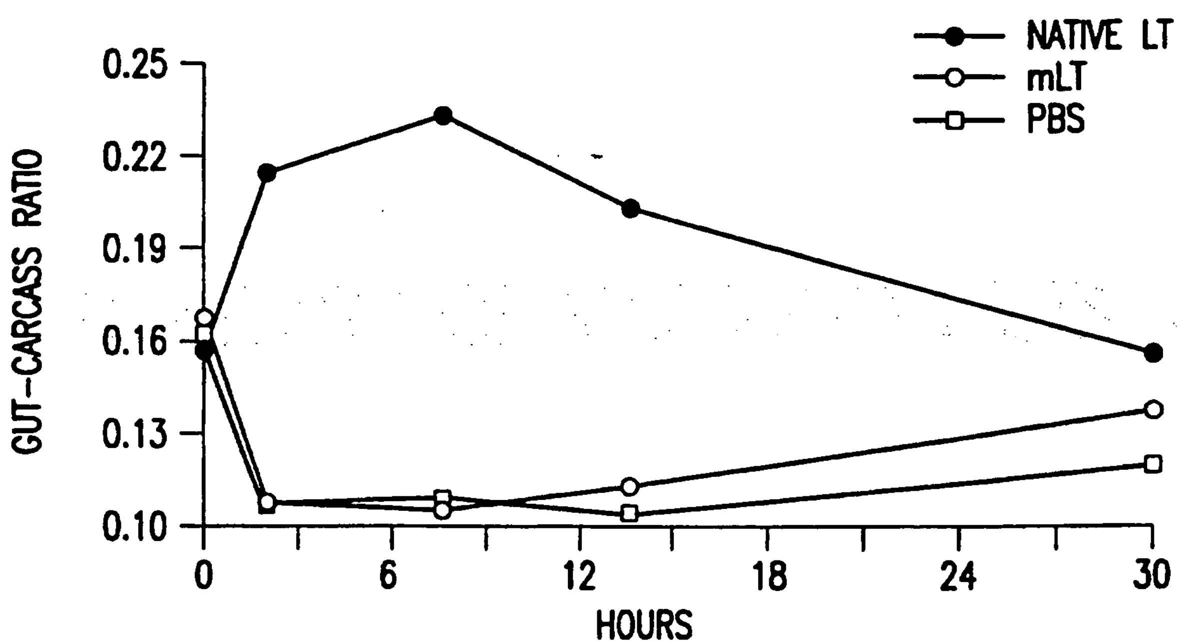
NATIVE LT
mLT
PBS
0.25
0.22
0.19
0.16
0.13
0.10
GUT–CARCASS RATIO
0
6
12
18
24
30
HOURS

FIG.3

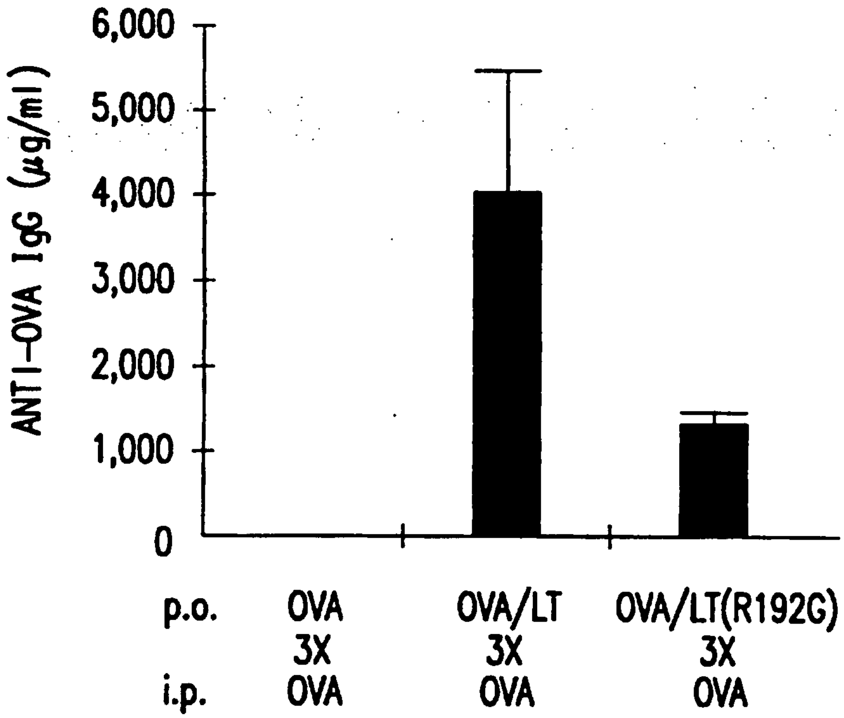
6,000
5,000
4,000
3,000
2,000
1,000
0
ANTI-OVA IgG (μg/ml)
p.o.
i.p.
OVA
3X
OVA
OVA/LT
3X
OVA
OVA/LT(R192G)
3X
OVA

FIG.4A

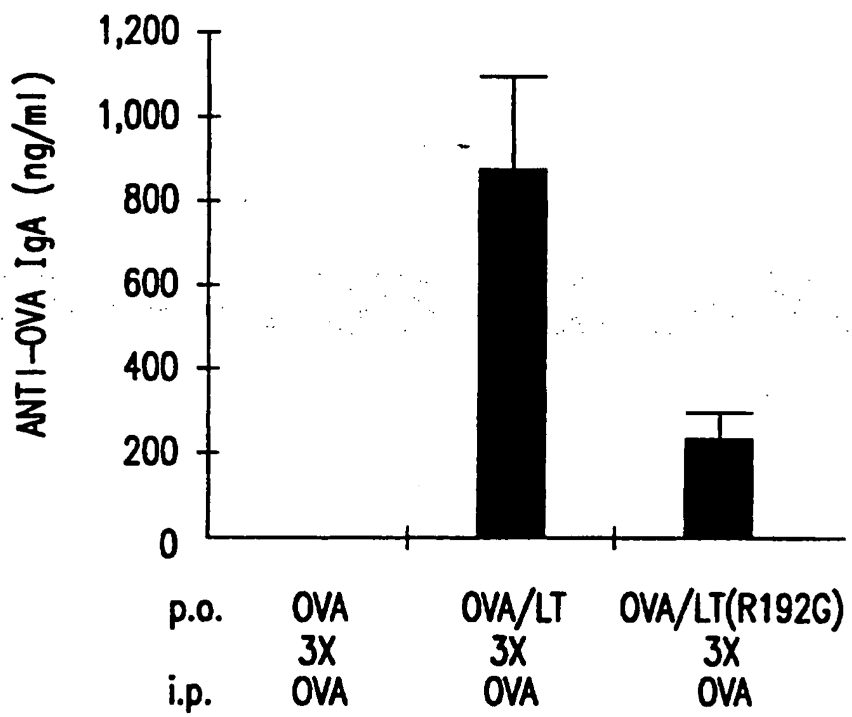
1,200
1,000
800
600
400
200
0
ANTI-OVA IgA (ng/ml)
p.o.
i.p.
OVA
3X
OVA
OVA/LT
3X
OVA
OVA/LT(R192G)
3X
OVA

FIG.4B

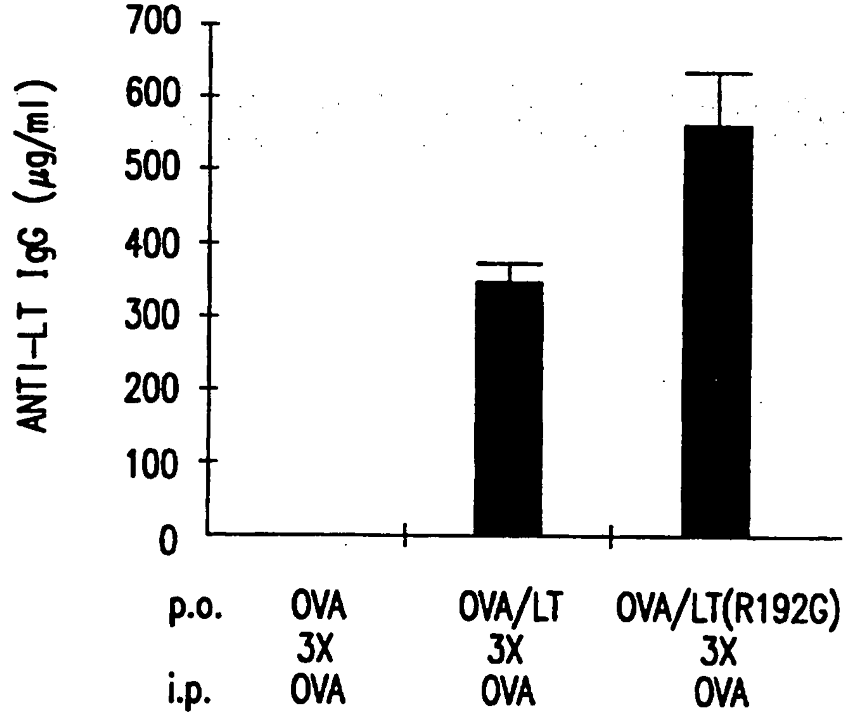
ANTI-LT IgG (μg/ml)
700
600
500
400
300
200
100
0
p.o.
i.p.
OVA
3X
OVA
OVA/LT
3X
OVA
OVA/LT(R192G)
3X
OVA

FIG.5A

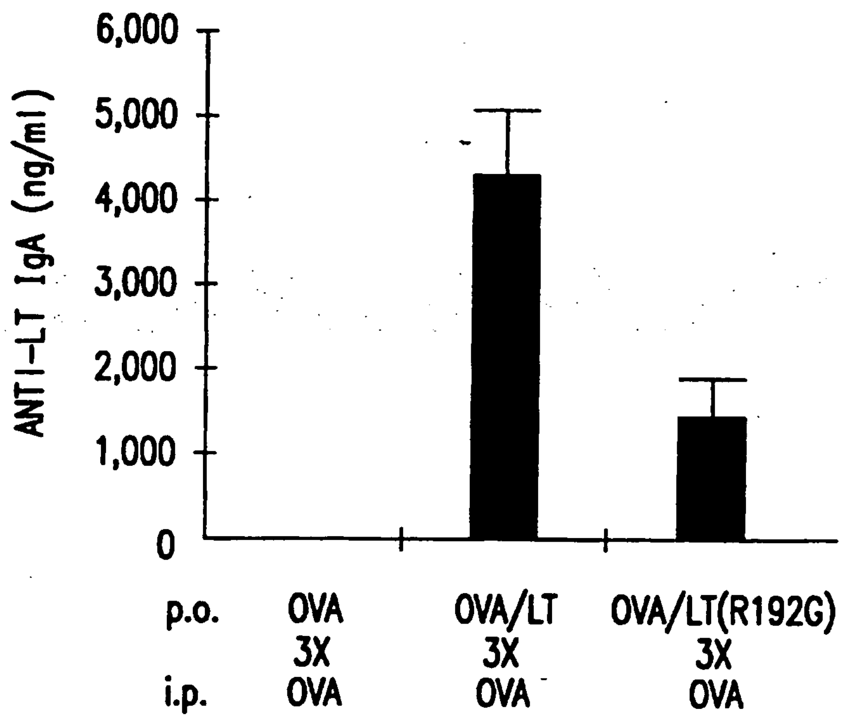
ANTI-LT IgA (ng/ml)
6,000
5,000
4,000
3,000
2,000
1,000
0
p.o.
i.p.
OVA
3X
OVA
OVA/LT
3X
OVA
OVA/LT(R192G)
3X
OVA

FIG.5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09005

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 39/02, 39/108
US CL :530/324, 868; 424/190.1, 236.1, 241.1, 257.1, 832
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324, 868; 424/190.1, 236.1, 241.1, 257.1, 832

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOTECHNOLOGY, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BACTERIOLOGY, Volume 170, No. 5, issued May 1988, K. Okamoto et al, "Effect of Substitution of Glycine for Arginine at Position 146 of the A1 Subunit on Biological Activity of *Escherichia coli* Heat-Labile Enterotoxin", pages 2208-2211, see entire document.	1-22
Y	EUR. J. IMMUNOL., Volume 22, issued 1992, N. Lycke et al, "The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity", pages 2277-2281, see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 OCTOBER 1995	13 NOV 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	LAURIE SCHEINER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF INFECTIOUS DISEASES, Volume 158, No. 2, issued August 198o, J. D. Clemens et al, "Cross-Protection by B Subunit-Whole Cell Cholera Vaccine Against Diarrhea Associated with Heat-Labile Toxin-Producing Enterotoxigenic *Escherichia coli*: Results of a Large-Scale Field Trial", pages 372-377, see entire document.	1-22
Y	VACCINE, Volume 6, issued June 1988, J.D. Clements et al, "Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens", pages 269-277, see entire document.	1-22
Y	INFECTION AND IMMUNITY, Volume 46, No. 2, issued November 1984, J. D. Clements et al, "Construction of a Potential Live Oral Bivalent Vaccine for Typhoid Fever and Cholera-*Escherichia coli*-Related Diarrheas", pages 564-569, see entire document.	1-22
Y	INFECTION AND IMMUNITY, Volume 40, issued May 1983, J. D. Clements et al, "Cloning and Molecular Characterization of the B Subunit of *Escherichia coli* Heat-Labile Enterotoxin", pages 653-658, see entire document.	1-22
Y	NATURE, Volume 288, issued 04 December 1980, W. S. Dallas et al, "Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin", pages 499-501, see entire document.	1-22
Y	INFECTION AND IMMUNITY, Volume 24, No. 3, issued June 1979, J.D. Clements et al, "Isolation and Characterization of Homogeneous Heat-Labile Enterotoxins with High Specific Activity from *Escherichia coli* Cultures", pages 760-769, see entire document.	1-22
Y	INFECTION AND IMMUNITY, Volume 22, No. 3, issued December 1978, J. D. Clements et al, "Demonstration of Shared and Unique Immunological Determinants in Enterotoxins from Vibrio cholerae and *Escherichia coli*", pages 709-713, see entire document.	1-22